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| (54) Title: POLYENE MACROLIDE PRE-LIPOSOMAL POWDERS<br>(57) Abstract<br><p>The present invention involves a process for producing fine powder suitable for the preparation of antifungal polyene macrolide-containing liposomes upon suspension in an aqueous solution. This process comprises the following steps. Quantities of polyene macrolide and phospholipids are dissolved respectively in a first solvent and a second solvent to form a first solution and a second solution. The first solution and the second solution are mixed in a desired ratio to form a mixture. The first solvent and the second solvent are then removed from the mixture, for example by evaporation, to form a residue. The residue is then dissolved in a third solvent comprising tertiary butanol and methylene chloride to form a third solution. The third solvent is then removed from the third solution to form a remnant. The remnant is then dissolved in a solvent consisting essentially of tertiary butanol to form a fourth solution. The fourth solution is then filtered through a filter having orifices of between about 0.05 and 0.5 micrometers in diameter to produce a filtrate. The filtrate is lyophilized to remove the tertiary butanol and a fine powder remains. This fine powder may be used to form polyene macrolide-containing liposomes by simple incubation or suspension in an aqueous solution.</p> |  |   |

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Polyene macrolide pre-liposomal powders.

# BACKGROUND OF THE INVENTION

The present invention relates to a composition of matter usable to form liposomes comprising antifungal  
5 polyene macrolides and the production thereof.

Clinical observations and animal experimental studies have added to the understanding of host-fungal interactions. It is becoming recognized that host defense  
10 against fungal disease is multifactorial and may vary, depending on the etiologic agent. The mechanisms of resistance are not well defined in most instances, but various innate barriers and cell-mediated immune responses seem to be of primary importance. Clearly, debilitation  
15 of innate defenses and of cell-mediated immune responses can increase an individual's susceptibility to severe fungal disease from opportunistic agents such as Cryptococcus neoformans and species of Candida and Aspergillus, as well as from fungal pathogens such as Histo-  
20 plasma capsulatum and Coccidioides immitis. The difficulty in gaining a complete understanding of the critical host defenses has been further complicated by many studies that show fungi may affect various host immune functions adversely. Although it is too early to evaluate the  
25 clinical importance of many of these experimental findings, investigators have demonstrated that fungi impair neutrophil function, induce IgE responses, and cause suppression of cell-mediated immune responses.

30 Host changes likely to be associated with increased susceptibility may be accidentally induced, as in traumatic injuries (such as burns or puncture wounds); self-induced, as in chronic alcoholism; naturally occurring, as in diabetes mellitus, various congenital immune deficien-

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cies, collagen diseases, lymphoreticular neoplastic disease, and other types of tumors; or iatrogenically induced by instrumentation (such as catheterization), surgical procedures (such as open heart surgery), or by  
5 use of cytotoxic drugs (as in an attempt to prevent graft rejection and to treat neoplastic disease), corticosteroid therapy, and long-term use of broad-spectrum antibiotics.

Chemical factors that aid resistance to fungal  
10 diseases are poorly defined. Knowledge of these substances is based primarily on circumstantial evidence at the clinical level and in vitro observations at the experimental level. Hormonally associated increases in lipid and fatty acid content on the skin occurring at  
15 puberty have been correlated with increased resistance to tinea capitis caused by the dermatophyte Microsporum audouinii, although pubescent changes are not the sole factors in resistance. Substances in serum, cerebrospinal fluid, and saliva may limit growth of Cryptococcus neoformans, and basic peptides in body fluids have been shown to  
20 inhibit Candida albicans.

Results of clinical and experimental studies indicate that C. albicans, C. neoformans, Aspergillus fumigatus,  
25 and C. immitis activate the alternative pathway of the complement cascade. Because of the polysaccharide nature of fungal cell walls, it is expected that all medically important fungi activate complement. Such activation may be important in defense against some mycoses; a positive  
30 correlation has been demonstrated between animals deficient in late-acting complement components (C3-C9) and increased susceptibility to fungi such as C. neoformans and C. albicans. Assuming that phagocytic cells are important in resistance to fungi, complement activation

may play a role by provoking an acute inflammatory response on generation of complement fragments C3a and C5a, and by coating the fungal elements with opsonic fragments C3b and C3d for ingestion by phagocytic cells.

5

The systemic mycoses of humans and other animals are caused by some fungi that are pathogenic and cause disease in the healthy host, and by other fungi (opportunistic pathogens) that are usually innocuous but cause disease in patients whose immune defenses are impaired. Some of these fungi may be saprophytes in nature (soil, bird droppings), whereas others are a part of the normal human flora (commensals). In no case are humans the solitary or necessary host.

15

An example of a soil saprophyte is Histoplasma capsulatum, which commonly causes infection in endemic areas; 80%-90% of adults react positively to histoplasmin in delayed cutaneous hypersensitivity tests. An example of an opportunistic pathogen is Candida albicans, normally present in the oral cavity, gastrointestinal tract, and probably the skin. In the patient with acute leukemia, however, C. albicans is commonly present in blood, causing a fulminant, usually fatal, septicemia. Other opportunistic infections are seen in patients with diabetic acidosis (mucormycosis) and Hodgkin's disease (for example, cryptococcosis and histoplasmosis). The pathogenesis of these mechanisms is obscure, but cell-mediated immunity seems to be essential for a good prognosis.

25  
30

Neither active vaccines nor passive immune serum immunization has been sufficiently successful to result in commercially available preparations.

Treatment of active disease may be symptomatic (for example, pain relief), sometimes surgical (resection of irretrievably damaged tissue and correction of hydrocephalus), and, most successfully, chemotherapeutic (Table 1). Among the chemotherapeutic agents commonly used are hydroxystilbamidine isethionate, amphotericin B, 5-fluorocytosine (Flucytosine), miconazole, and ketoconazole. Response to these drugs varies according to the fungus, type of disease, and course of illness. For example, response is good in most B. dermatitidis infections, but is poor in most diseases caused by A. fumigatus. Response is better for skin lesions caused by B. dermatitidis than for meningitis due to C. immitis; response is better in chronic cryptococcosis than in fulminant candidiasis. Table 1 shows a listing of some systemic mycoses and generally accepted chemotherapeutic agents.

TABLE 1

## 5 CHEMOTHERAPEUTIC AGENTS FOR SYSTEMIC MYCOSES

|    | Disease   | First Choice                  | Second Choice                      |
|----|---|-------------------------------|------------------------------------|
| 10 | Aspergillosis   | Amphotericin B                | Ketoconazole                       |
|    | Blastomycosis   | Amphotericin B                | Hydroxystilbamidine<br>isethionate |
|    | Candidiasis   | Amphotericin B                | Flucytosine or<br>ketoconazole     |
| 15 | Coccidioidomycosis  | Amphotericin B                | Ketoconazole                       |
|    | Cryptococcosis  | Amphotericin B<br>Flucytosine | Either drug alone*                 |
|    | Histoplasmosis  | Amphotericin B                | Ketoconazole*                      |
| 20 | Mucormycosis  | Amphotericin B                | Miconazole*                        |
|    | Paracoccidioidomycosis  | Amphotericin B                | Sulfonamides,<br>Ketoconazole*     |
| 25 | *Depending on minimal inhibitory concentration necessary<br>for the fungus. |                               |                                    |

Infection is the cause of death in 51% of patients with lymphoma and 75% of patients with leukemia. Although bacteria are the causative organisms of many such infections, fungi account for 13% of the fatal infections in patients with lymphoma and for more than 20% of patients with leukemia. The fungus Candida albicans causes more than 80% of these infections, and Aspergillus spp. is also a frequent cause of such infections. In addition, fungal infection is a major cause of morbidity and mortality in patients with congenital and acquired deficiencies of the immune system. Much concerted effort has been expended in search of agents useful in treating fungal infections of humans. As a result, many compounds have been isolated and shown to have antifungal activity, but problems associated with solubility, stability, absorption, and toxicity have limited the therapeutic value of most of them in human infections. The most useful antifungal antibiotics fall into one of two categories: those that affect fungal cell membranes and those that are taken up by the cell and interrupt vital cellular processes such as RNA, DNA, or protein synthesis. Table 2 lists some useful antifungal agents and their mechanisms of action.



TABLE 2

## SOME USEFUL ANTIFUNGAL AGENTS, THEIR CHEMICAL CLASSIFICATION, AND THEIR MECHANISMS OF ACTION

|    |                      |   |  |
|----|----------------------|---|--|
| 5  |                      |   |  |
|    | <b>Class</b>         | <b>Compounds</b>                                    | <b>Mechanism</b>   |
| 10 | <b>Polyene</b>       | <b>Amphotericin B<br/>Nystatin</b>                  | <b>Interacts with sterols (ergosterol) in fungal cell membrane, rendering cells selectively permeable to the outflow of vital constituents, e.g. potassium</b>                       |
| 15 |                      |   |  |
| 20 | <b>Imidazole</b>     | <b>Miconazole<br/>Clotrimazole<br/>Ketoconazole</b> | <b>Inhibits demethylation of lanosterol thus preventing formation of ergosterol, a vital component of fungal cell membrane; also has a direct lethal effect on fungal cells</b>      |
| 25 |                      |   |  |
| 30 | <b>Pyrimidine</b>    | <b>5-Fluorocytosine</b>                             | <b>Is taken up and deaminated by susceptible cell to form 5-fluorouracil, which in turn inhibits RNA synthesis; also thought to inhibit thymidylate synthetase and DNA synthesis</b> |
| 35 |                      |   |  |
| 40 | <b>Griseofulvin</b>  | <b>Griseofulvin</b>                                 | <b>Binds to tubulin and inhibits microtubule assembly</b>  |
| 45 | <b>3-Arylpyrrole</b> | <b>Pyrrolnitrin</b>                                 | <b>Appears to inhibit terminal electron transport between succinate and NADH and coenzyme Q</b>  |
| 50 | <b>Glutaramide</b>   | <b>Cycloheximide</b>                                | <b>Inhibits protein synthesis at 80S ribosomal level preventing transfer of aminoacyl tRNA to the ribosome</b>   |

The polyene macrolide antibiotics are secondary metabolites produced by various species of Streptomyces. Several common features of these compounds are useful in classifying the more than 80 different polyenes that have been isolated. All are characterized by a macrolide ring, composed of 26-38 carbon atoms and containing a series of unsaturated carbon atoms and hydroxyl groups. These features of the molecule contribute to the polyenes' amphipathic properties (those relating to molecules containing groups with different properties, for example, hydrophilic and hydrophobic). The ring structure is closed by the formation of an internal ester or lactone bond (Figure 1). The number of conjugated double bonds vary with each polyene, and the compounds are generally classified according to the degree of unsaturation.

Toxic effects of polyene macrolides appear to be dependent on binding to cell membrane sterols. Thus, they bind to membranes of fungus cells as well as to those of other eukaryotic cells (human, plant, and protozoa), but not to bacterial cell membranes, which do not contain membrane sterols. The interaction of polyene macrolides with mammalian and fungal membrane sterols results in transmembrane channels that allow the leakage of intracellular components leading to cell deaths.

The usefulness of an antibiotic is usually measured by the differential sensitivity of the pathogen and host. Two polyene macrolides agents, nystatin and amphotericin B, are relatively specific for fungi and have thusfar proven to have therapeutic usefulness in humans. The relative specificity of these two polyene macrolides may be based on their greater avidity for ergosterol, the

principal sterol of fungal membranes, compared to cholesterol, the principal sterol of human cell membranes.

Amphotericin B is a heptaene macrolide with seven  
5 resonating carbon bonds. The compound was first isolated  
from broth filtrates of S. nodosum in 1956. Like other  
polyene macrolide antibiotics, amphotericin B is insoluble  
in water. The problem of its solubility has been circum-  
10 vented by combining the antibiotic with sodium deoxycho-  
late and sodium phosphate and hydrating the mixture with  
sterile water or saline. Amphotericin B is the polyene  
antibiotic thusfar most sufficiently nontoxic to humans  
that it has been used parenterally at effective doses  
against various fungi.

15 Nystatin, first isolated from S. noursei, is struc-  
turally related to amphotericin B, but is not classified  
as a heptaene because the conjugated portion of the ring  
is interrupted and thus forms a tetraene and a diene.  
20 Tolerated well both orally and topically, the drug is not  
available for intravenous use because of its presumed high  
toxicity and aqueous insolubility. Nystatin is available  
as oral tablets (500,000 units) or as an ointment for  
topical use (100,000 units/g). It is used in the manage-  
25 ment of cutaneous and mucocutaneous candidiasis.

It has recently been shown that the encapsulation of  
certain drugs in liposomes before administration to the  
patient can markedly alter the pharmacokinetics, tissue  
30 distribution, metabolism and therapeutic efficacy of these  
compounds. Liposomes may be defined as lipid vesicles  
which are formed spontaneously on addition of an aqueous  
solution to a dry lipid film. Further, the distribution  
and pharmacokinetics of these drugs can be modified by

altering the lipid composition, size, charge and membrane fluidity of the liposome in which they are encapsulated.

Recently, liposomes have been used as carriers of  
5 amphotericin B for treatment of murine leishmaniasis (New, R.R.C., et al., "Antileishmanial Activity of Amphotericin and Other Antifungal Agents Entrapped in Liposomes." J. Antimicrob. Chemother., Vol. 8 (1981), pp. 371-381), histoplasmosis (Taylor, R.L., et al., "Amphotericin B in  
10 Liposomes: A Novel Therapy for histoplasmosis." Am. Rev. Respir. Dis., Vol. 125 (1982), pp. 610-611), cryptococcosis (Graybill, J.R., et al., "Treatment of Murine Cryptococcosis with Liposome-Associated Amphotericin B." J. Infect. Dis., Vol. 145 (1982), pp. 748-752). and candidiasis  
15 (Tremblay, C., et al., "Comparative Efficacy of Amphotericin B (AMB) and Liposomal AMB (lip-AMB) in Systemic Candidiasis in Mice." Abstr. 1983 ICAAC, No. 755 (1983), p. 222). Liposome-encapsulated Amphotericin B has also been used for treatment of coccidioidomycosis in the  
20 Japanese macaque (Graybill, J.R., et al., "Treatment of Coccidioidomycosis (cocci) in Primates Using Liposome Associated Amphotericin B (Lipo-AMB)." Abstr. 1982 ICCAC, No. 492 (1982), p. 152).

25 The treatment of fungal infections remains a major problem in spite of the availability of effective anti-fungal drugs such as the polyenes. Most of the available polyene antibiotics have toxic side effects that limit their clinical application. Nystatin, a tetraene-diene  
30 polyene macrolide antibiotic, has high hydrophobicity, which has precluded its effective systemic administration. It has been used as suspensions prepared in various ways and administered to the patients orally. However, these studies have generally failed to document a beneficial

effect of nystatin administration against systemic fungal infections.

The present inventors have recently demonstrated that  
5 liposome-encapsulated amphotericin B may be used to treat  
experimental murine candidiasis (Lopez-Berestein et al.,  
J. Infect. Dis., Vol. 150, pp 278-283 (1984) and in the  
treatment of fungal infections in patients with leukemia  
and lymphoma (Lopez-Berestein et al., J. Infect. Dis.,  
10 Vol. 151, pp 704-71- (1985).

#### SUMMARY OF THE INVENTION

15 The present invention involves a process for  
producing fine powder suitable for the preparation of  
antifungal polyene microlide-containing liposomes upon  
suspension in an aqueous solution. This process comprises  
the following steps. Quantities of polyene macrolide and  
20 phospholipids are dissolved respectively in a first  
solvent and a second solvent to form a first solution and  
a second solution. The first solution and the second  
solution are mixed in a desired ratio to form a mixture.  
The first solvent and the second solvent are then removed  
25 from the mixture, for example by evaporation, to form a  
residue. The residue is then dissolved in a third solvent  
comprising tertiary butanol and methylene chloride to form  
a third solution. The third solvent is then extracted by  
evaporation from the third solution to form a remnant.  
30 The remnant is then dissolved in a solvent consisting  
essentially of tertiary butanol to form a fourth solution.  
The fourth solution is then filtered through a filter  
having orifices of between about 0.05 and 0.5 micrometers  
in diameter to produce a filtrate. The filtrate is

lyophilized to remove the tertiary butanol and a fine powder remains. This fine powder may be used to form polyene macrolide-containing liposomes by simple incubation or suspension in an aqueous solution.

5

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

A stable powder suitable for the direct preparation of liposome-incorporated antifungal polyene macrolides may be made by a process of the present invention. While the conditions described herein are specifically applicable to nystatin and amphotericin B, other polyene macrolide antifungals may be likewise used, but with minor modifications of procedure apparent to those skilled in the art upon a minimal amount of experimentation.

The process for pre-liposomal polyene macrolide powder formation of the present invention involves dissolution of an antifungal polyene macrolide such as nystatin or amphotericin B in a first organic solvent such as methanol to form a first solution. Phospholipids are dissolved in a second organic solvent such as, for example, chloroform, to form a second solution. The first solution and the second solution are mixed to form a first mixture having a ratio of antifungal polyene macrolide to phospholipid between about 1:5 and about 1:50, preferably of about 1:10. The organic solvents are removed from the mixture, for example, by solvent evaporation under reduced pressure and at a temperature between about 35°C and about 45°C, until a residue such as a dry film is formed. The residue is then dissolved in a quantity of a third organic solvent such as a mixture of tertiary butanol and methylene chloride in a ratio between about 2:1 (preferred for

nystatin) and about 1:40 (preferred for amphotericin B) and the solvent evaporated to leave a remnant. The remnant is dissolved in a solvent consisting essentially of tertiary butanol to form a fourth solution which is  
5 warmed, if necessary for clarification, and passed through a filter having orifices of between about 0.05 and 0.5 micrometers ( $\mu$ m) in diameter. If warming is desired to clarify the fourth solution, particularly with amphotericin B, the warming is preferably to a temperature  
10 between about 50°C and about 70°C. The filtrate is subjected to freezing, for example, with dry ice-acetone. The frozen material is then lyophilized until essentially all solvent is removed. After lyophilization, a fine pre-liposomal polyene macrolide powder is produced. This  
15 powder is readily and stably stored under commonly available dry and cool storage conditions.

The above-described pre-liposomal polyene macrolide powder may be easily used to reconstitute a liposome  
20 suspension according to the following general procedure. The powder is added to an aqueous solution such as pyrogen-free saline, and allowed to incubate at 25°C to 45°C for 1-10 minutes for a liposome suspension to form. Polyene macrolide content may be measured by dissolution  
25 of the liposomes in methanol and monitoring of optical density at a wavelength characteristic for polyene macrolide absorption.

Representative, suitable phospholipids in the present  
30 invention are phosphatidylcholine, both naturally occurring and synthetically prepared, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, sphingolipids, phosphatidylglycerol, sphingomyelin, cardiolipin, glycolipids, gangliosides, cerebrosides and the like used

either singularly or intermixed such as in soybean phospholipids.

More particularly useful phospholipids include egg  
5 phosphatidylcholine, dilaurylphosphatidylcholine, dimy-  
ristoylphosphatidylcholine, dipalmitoylphosphatidyl-  
choline, distearoylphosphatidylcholine, 1-myristoyl-2-  
palmitoylphosphatidylcholine, 1-palmitoyl-2-myristoyl  
phosphatidylcholine, 1-stearoyl-2-palmitoyl phosphatidyl-  
10 choline, dioleoylphosphatidylcholine, dilauryloylphospha-  
tidylglycerol, dimyristoylphosphatidylglycerol,  
dipalmitoylphosphatidylglycerol, distearoylphosphatidyl-  
glycerol, dioleoylphosphatidylglycerol, dimyristoyl  
phosphatidic acid, dipalmitoyl phosphatidic, dimyristoyl  
15 phosphatidylethanolamine, dipalmitoyl phosphatidyl-  
ethanolamine, dimyristoyl phosphatidylserine, dipalmitoyl  
phosphatidylserine, brain phosphatidylserine, brain  
sphingomyelin, dipalmitoyl sphingomyelin, and distearoyl  
sphingomyelin.

20

The lipid composition of both the initial powdered  
composition of matter and the resultant liposomes, formed  
in accordance with the present method, is normally the  
same. Where the resultant liposomes are intended for  
25 in vivo applications (such as drug delivery), then it is  
normally desirable that the lipid composition have a  
transition temperature below body temperature. Liposomes  
composed of phospholipids which have transition  
temperatures below the characteristic gel-liquid  
30 crystalline phase transition temperature of biological  
membranes, i.e. about 37°C, are considered fluid and those  
which have transition temperature above 37°C are  
considered solid. Another consideration in selecting the  
composition of lipid or lipids for liposome applications



is that alkyl-ether linked lipids (rather than ester linked) are more stable to hydrolysis, and hence alkyl-ether linked lipids for the resultant liposomes may be particularly desirable for therapeutic application.

5

In addition, other lipid-like substances such as steroids, cholesterol, aliphatic amines or acids such as long chain aliphatic amines or carboxylic acids, long chain sulfates and phosphates, dicetyl phosphate, butylated hydroxytoluene, tocopherol, and isoprenoid compounds may be intermixed with the phospholipid components to confer certain desired and known properties on the initial liposomes and hence the resultant liposomes. Further, synthetic phospholipids containing either altered aliphatic portions, such as hydroxyl groups, branched carbon chains, cycloderivatives, aromatic derivatives, ethers, amides, polyunsaturated derivatives, halogenated derivatives, or altered hydrophilic portions containing carbohydrate, glycol, phosphate, phosphonate, quaternary amine, sulfate, sulfonate, carboxy, amine, sulfhydryl, imidazole groups and combinations of such groups, can be either substituted or intermixed with the phospholipids.

25 The antifungal polyene macrolides of the present invention include nystatin, amphotericin B, partricin and derivatives thereof such as methyl esters.

30 These examples are presented to illustrate preferred embodiments and utilities of the present invention and are not meant to limit the present invention unless otherwise stated in the claims appended hereto.

## EXAMPLE 1

Preparation and Use of a Pre-Liposomal  
Nystatin Powder (L-Nys)

5

A solution of 25 mg nystatin in 25 ml methanol was mixed with a solution of 175 dimyristoylphosphatidylcholine (DMPC) and 75 mg dimyristoylphosphatidylglycerol (DMPG) in 10 ml chloroform. The DMPC:DMPG ration was 7:3 and the nystatin:DMPC+DMPG ration was 1:10. The organic solvents were then evaporated at 40°C under partial vacuum in a rotary evaporator until a dried lipid film was formed. Thirty ml of 2:1 mixture of tertiary butanol and methylene chloride were added to dissolve the dried lipid film. The organic were then evaporated from the solution at 40°C and under partial vacuum to form a lipid residue. The lipid residue was dissolved in tertiary butanol and the solution passed through a 0.2 um filter. The nystatin concentration was measured from an aliquot of the filtrate. The filtrate was frozen by immersion of a container in dry ice-acetone. The frozen material was subjected to overnight lyophilization and a fine pre-liposomal nystatin powder produced.

25

A 100 mg sample of the fine powder (containing about 10 mg nystatin) was suspended with 10 ml of pyrogen-free saline. When the powder suspension was warmed at 40°C for 2-5 minutes, liposomes were formed therein. As determined by microscopic examination, the suspended materials were 100% liposomes were formed therein. As determined by microscopic examination, the suspended materials were 100% liposomes and no crystals were found. The suspension was centrifuged at 20,000 rpm (40,700 x g) for one hour and the resultant pellet removed and resuspended in saline.

30

The nystatin remaining in the resuspended pellet was determined to be 70-80 percent of the original amount added, by dissolution in methanol and measurement of optical density at 306 nm. The encapsulation efficiency of the liposomes, as measured after the filtration step, was observed to be > 99%. (No detectable free drug was left after formation of liposomes from the powder). The resuspended pellet was a liposome preparation substantially free of soluble lipids or other materials and was suitable for clinical administration.

#### EXAMPLE 2

##### 15                    Preparation and Use of a Pre-Liposomal Amphotericin B Powder

Amphotericin B in methanol and phospholipids (DMPC:DMPG, 7:3) in chloroform were mixed together in a ratio of 1:10. The organic solvents were then evaporated at 40°C using a rotary evaporator under vacuum.

Tertiary butanol and methylene chloride in a 1:30-40 ratio were added to solubilize the dried lipid film. The organic solvents were then evaporated.

The residue in the flask was then dissolved in tertiary butanol, warmed to temperatures above 52°C, and filtered through a 0.2 um filter. An aliquot from this filtrate was taken to determine the amphotericin B concentration.

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The above mixture was then frozen (using dry ice with acetone) and lyophilized overnight. A fine powder was obtained.

5        The powder obtained as described above was suspended in pyrogen-free saline. The liposomes did not form until the suspension was warmed in a water bath at about 40°C for about 2-5 minutes. The suspension then formed 100% liposomes (no crystals), as they appeared under a micro-  
10 scope. The suspension was centrifuged at 20,000 rpm for one hour and the pellet removed and resuspended in saline. An aliquot was taken from this final suspension and the amount of amphotericin B incorporated into liposomes quantitated by dissolving in methanol and measuring O.D.  
15 at 405 nm. The encapsulation efficiency of drug from the powder to liposomes was 99-100%.

\* \* \* \* \*

20

Changes may be made in the elements and methods described herein or in the steps or the sequence of steps of the method described herein without departing from the  
25 concept and scope of the invention as defined in the following claims.

WHAT IS CLAIMED IS:

1. A process for producing a powder which forms  
5 liposomes comprising an antifungal polyene macrolide upon  
suspension in an aqueous solution, said process comprising  
the steps of:
  - 10 (a) dissolving antifungal polyene macrolide and  
phospholipids in a quantity of first organic  
solvent and a quantity of second organic solvent  
respectively, to form a first solution and a  
second solution;
  - 15 (b) mixing the first solution and the second solution  
to form a mixture;
  - (c) removing the first organic solvent and the second  
organic solvent from the mixture to form a  
20 residue;
  - (d) dissolving the residue in a quantity of a third  
organic solvent to form a third solution;
  - 25 (e) extracting the third organic solvent from the third  
solution to leave a remnant;
  - (f) forming a fourth solution by dissolving the remnant  
in a solvent consisting essentially of tertiary  
30 butanol;
  - (g) passing the fourth solution through a filter having  
orifices with diameters of between about 0.1 nm  
and about 0.5 nm to produce a filtrate; and

(h) lyophilizing the filtrate to remove the solvent consisting essentially of tertiary butanol.

5 2. A composition of matter produced essentially by the process of claim 1.

3. A composition of matter produced by a process  
10 comprising the steps of:

(a) dissolving antifungal polyene macrolide and phospholipids in a quantity of first organic solvent and a quantity of second organic solvent  
15 to form respectively a first solution and a second solution;

(b) mixing the first solution and the second solution to form a first mixture;  
20

(c) removing the first organic solvent and the second organic solvent to form a residue;

(d) dissolving the residue in a quantity of a third organic solvent to form a third solution;  
25

(e) extracting the third organic solvent from the third solution to leave a remnant;

30 (f) forming a fourth solution by dissolving the remnant in a solvent consisting essentially of tertiary butanol;

- (g) passing the fourth solution through a filter having orifices with diameters of between about 0.1 nm and about 0.5 nm to produce a filtrate; and
- 5 (h) lyophilizing the filtrate to remove the solvent consisting essentially of tertiary butanol.
4. The process of claim 1 or composition of matter of  
10 claim 3 wherein the antifungal polyene macrolide is nystatin, amphotericin B, partricin or a derivative thereof.
- 15 5. The process of claim 1 or composition of matter of claim 3 wherein the antifungal polyene macrolide is nystatin or amphotericin B.
- 20 6. The process of claim 1 or composition of matter of claim 3 wherein the antifungal polyene macrolide is amphotericin B.
- 25 7. The process of claim 1 or composition of matter of claim 3 wherein the antifungal polyene macrolide is nystatin.
- 30 8. The process of claim 1 or the composition of matter of claim 3 wherein the phospholipids are one or more of phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, sphingomyelin and phosphatidic acid.

9. The process of claim 1 or the composition of matter of claim 3 wherein the phospholipids comprise DMPC and DMPG.

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10. The process of claim 1 or the composition of matter of claim 3 wherein the phospholipids consist essentially of DMPC and DMPG in 7:3 ratio.

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11. The process of claim 1 or the composition of matter of claim 3 wherein the first solvent is methanol.

15 12. The process of claim 1 or the composition of matter of claim 3 wherein the second solvent is chloroform.

13. The process of claim 1 or the composition of matter  
20 of claim 3 wherein step (b) is defined further as:

mixing the first solution and the second solution to  
form a first mixture having a ratio of anti-  
fungal polyene macrolide to phospholipid between  
25 about 1:5 and about 1:50.

14. The process of claim 1 or the composition of matter  
of claim 3 wherein step (b) is defined further as:

30

mixing the first solution and the second solution to  
form a first mixture having a ratio of anti-  
fungal polyene macrolide to phospholipid of  
about 1:10.



15. The process of claim 1 or the composition of matter of claim 3 wherein step (c) is defined further as:

5 removing the first solvent and the second solvent from the first mixture by subjecting the first mixture to solvent evaporation under reduced pressure and at a temperature between about 35°C and about 45°C.

10

16. The process of claim 1 or the composition of matter of claim 3 wherein, prior to the passing step, the fourth solution is clarified by warming to between about 50°C and about 70°C.

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17. The process of claim 1 or the composition of matter of claim 3 wherein the third organic solvent comprises tertiary butanol and methylene chloride.

20

18. The process of claim 1 or the composition of matter of claim 3 wherein the third organic solvent comprises tertiary butanol and methylene chloride in a ratio between  
25 about 2:1 and about 1:40.

19. The process of claim 1 or the composition of matter of claim 3 defined further wherein the filter has orifices  
30 of about 0.2 nm.

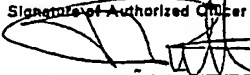
20. The process of claim 1 or the composition of matter of claim 3 wherein the antifungal polyene macrolide and

phospholipids are in a ratio of between about 1:5 and about 1:20.

- 5 21. The process of claim 1 or the composition of matter of claim 3 wherein the antifungal polyene macrolide and phospholipids are in a ratio of about 1 to 10.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03652

|   |  |                                     |
|---|--|-------------------------------------|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *  |  |                                     |
| According to International Patent Classification (IPC) or to both National Classification and IPC   |  |                                     |
| IPC <sup>4</sup> : A 61 K 9/50; A 61 K 31/71  |  |                                     |
| <b>II. FIELDS SEARCHED</b>  |  |                                     |
| Minimum Documentation Searched <sup>7</sup>   |  |                                     |
| Classification System   | Classification Symbols   |                                     |
| IPC <sup>4</sup>  | A 61 K   |                                     |
| Documentation Searched other than Minimum Documentation<br>to the extent that such Documents are included in the Fields Searched <sup>8</sup>   |  |                                     |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> *   |  |                                     |
| Category <sup>9</sup>   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup> | Relevant to Claim No. <sup>13</sup> |
| A   | US, A, 4663167 (LOPEZ-BERESTEIN et al.)<br>5 May 1987<br>see column 5, lines 1-25<br>--                        |                                     |
| A   | FR, A, 2390159 (ICI)<br>8 December 1978<br>see page 6, example 1; claims<br>--                                 |                                     |
| A   | EP, A, 0087993 (PARFUMS CHRISTIAN DIOR)<br>7 September 1983<br>see pages 25,26, example 12; claims<br>-----    |                                     |
| <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> |  |                                     |
| <b>IV. CERTIFICATION</b>  |  |                                     |
| Date of the Actual Completion of the International Search   | Date of Mailing of this International Search Report  |                                     |
| 17th February 1989  | 16 MAR 1989  |                                     |
| International Searching Authority   | Signature of Authorized Officer  |                                     |
| EUROPEAN PATENT OFFICE  | <br>P.C.G. VAN-DER PUTTEN  |                                     |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8803652

SA 25325

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